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Influence of Different Substrates on the Production of a Mutant Thermostable Glucoamylase in Submerged Fermentation

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Abstract Three mutations, Ser54→Pro, Thr314→Ala, and His415→Tyr, were identified in *Aspergillus awamori* glucoamylase gene expressed by *Saccharomyces cerevisiae*. The mutant glucoamylase (GA) was substantially more thermostable than a wild-type GA at 70 °C, with a 3.0 KJ mol^{-1} increase in the free energy of thermo-inactivation. The effect of starch from different botanical sources on the production of this GA was measured in liquid fermentation using commercial soluble starch, cassava, potato, and corn as the carbon source. The best substrate for GA production was the potato starch showing an enzymatic activity of 6.6 U/mL. The commercial soluble starch was also a good substrate for the enzyme production with 6.3 U/mL, followed by cassava starch and corn starch with 5.9 and 3.0 U/mL, respectively. These results showed a significant difference on GA production related to the carbon source employed. The mutant GA was purified by acarbose–Sepharose affinity chromatography; the estimated molecular mass was 100 kDa. The mutant GA exhibited optimum activity at pH 4.5 and an optimum temperature of 65 °C.

Keywords Mutant glucoamylase · Thermostable enzyme · Production · Purification · Corn starch · Potato starch · Cassava starch · Thermo-inactivation

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Introduction

Starch is the most abundant form of polysaccharide storage in plants. It constitutes an inexpensive source for the production of syrups containing glucose and maltose which are widely used in food industries [1, 2].

Glucoamylase (GA; α-1,4-glucan glucohydrolase, amyloglucosidase, EC 3.2.1.3) is an important enzyme which hydrolyzes α-1,4-glycosidic bonds from the non-reducing ends of starch, resulting in the production of glucose. It also has the ability to hydrolyze α-1,6-linkages, which also has glucose as the end-product. It is produced by a variety of fungal species, although commercial supplies are primarily obtained from the genus *Aspergillus* and *Rhizopus* due to the fact that their enzymes display low transglycosylation activity and their ability to obtain near 100% yields of glucose from starch hydrolysis [3]. These enzymes are generally recognized as safe by the Food and Drug Administration [4].

The structure of the enzyme has two functional domains: an N-terminal catalytic domain in an $(\alpha/\alpha)_6$ barrel structure and a C-terminal starch-binding domain in a β-sheet structure. The two functional domains are linked by a heavily *O*-glycosylated linker region [5, 6].

GA has many applications in industry, being used in the brewing of low-calorie beer and in whole grain hydrolysis for the alcohol industry, but the most important application of this enzyme is dextrose production, especially in the production of high-glucose syrups [4].

The conversion of starch to sugars is one of the most important biotechnological processes [4, 7]. Conventionally, conversion of starch to glucose requires a two-step process, namely, liquefaction and saccharification. The starch slurry is first liquefied at 105 °C for 5 min and then at 95 °C for 1 h using thermostable α-amylase. GA is used in the second step to convert the dextrin into glucose monomers. This step involves cooling the dextrin to 60 °C, the operational temperature of GA. At this temperature, the reaction can take several days (48–72 h) to be completed [5]. GA only efficiently catalyzes the saccharification reaction within a relatively narrow range of temperatures because its catalytically active conformation changes at high temperatures. This low thermostability limits its use in industrial processes where prolonged incubation at high temperatures is required. Higher operational temperatures cannot only accelerate the reaction rate and, consequently, shorten the process time but can also prevent microbial contamination and reduce the viscosity of the reaction mixture. Therefore, the development of a more thermostable GA would greatly contribute to the starch saccharification process [8, 9].

In recent years, there has been a keen interest in the production of heterologous proteins by recombinant DNA technology, and numerous approaches have successfully been taken to improve the thermostability of GA by site-directed and random mutagenesis. The application of yeast cells to the production of foreign proteins offers several advantages. For example, many foreign proteins are produced in soluble form in yeast cells, and post-translation modifications generally occur, contributing to the enzyme stability [10].

Fermentation studies have shown that both nutrient source and the type of fermentation can affect the enzyme's properties [11] and that the effects of complex medium and different nitrogen sources also affect the stability of the plasmid in more microorganisms [12]. GA secretion is greatly dependent on the carbon source used [13].

In previous work, an *Aspergillus awamori* wild-type GA expressed in *Saccharomyces cerevisiae* was produced by submerged fermentation in starches from different sources and physicochemically characterized [14]. Then, the wild-type GA gene was submitted to a mutagenic polymerase chain reaction, obtaining a mutant GA with improved thermostability

[15]. In the present work, the amino acid substitution in the mutant was determined. The thermostable mutant was purified and the physical, chemical, and thermodynamic parameters of this enzyme were characterized .The production of GA on starches from different sources was also investigated.

Material and Methods

Microorganism

S. cerevisiae strain C468 (α *leu*2-3 *leu* 2-112 *his*3-11 *his* 3-15 *mal*$^-$) containing the plasmid YEpPM18, a yeast episomal expression vector containing the wild-type GA cDNA from *A. awamori*. The wild-type *A. awamori* GA gene was randomly mutated by PCR mutagenesis and transformed into yeast by in vivo recombination using vector YEpPM18. Plate screening of transformed colonies identified one mutated GA that was more thermostable than the wild type [15].

Sequencing of Mutant Genes

Plasmid YEpPM18 containing the mutated GA gene was isolated from the transformed yeast by the Hoffman and Winston method [16]. Competent *Escherichia coli* DH 5α were transformed by electroporation with this preparation; then, ampicillin-resistant transformants were selected. A single transformant colony of pPM18/DH5α was inoculated into LB + ampicillin; plasmid DNA containing the mutant gene was produced overnight and then purified using the Gene Jet™ Plasmid Miniprep kit (Fermentas). The gene was sequenced by Macrogen (Korea) using seven primers 5′CCCTGAGCGGCCTCGTCTGCA3′, 5′ CTCCGCCCAGGCAATTGTCCA 3′, 5′ CGATTGCTGTGCAACACCGCG 3′, 5′ TCAGTG ACAGCGAGGCTGTTG 3′, 5′ CGCAAGCAACGGCTCCATGTC 3′, 5′ACCAGCAAGAC CACCGCGACT3′, 5′ACACCGTTCCTCAGGCGTGCG 3′.

Culture Medium and Enzyme Production

Starches from four different botanic sources were used to produce the mutant GA under submerged fermentation. The culture medium and the growth conditions for GA production have been described previously [14].

Assay of GA Activity

The glucose liberated from starch by GA was estimated by the peroxidase/glucose oxidase enzymatic method described by Bergmeyer and Bernt [17], with appropriate modifications [14]. One unit of GA was defined as the amount of enzyme required to release 1 μmol of glucose per minute under the assay conditions, and activity was expressed as units per milliliter. The activity was also expressed as units per milligram proteins.

Microbial Biomass Quantification

The washed cells were dried to constant weight at 60 °C and their biomass was expressed as milligrams per milliliter, as previously described [14].

Protein Determination

The protein content of the enzyme solution was measured by the Hartree–Lowry method [18] using bovine serum albumin as the standard.

Purification

A submerged 120 h culture of *S. cerevisiae* was centrifuged at 10,000×*g* for 10 min and the supernatant was used as GA extract. The enzymatic extract was concentrated and diafiltrated against a buffer of 0.5 M NaCl/0.1 M NaOAc, pH 4.5, using an Amicon S1 spiral ultrafiltration cartridge. The concentrated enzymatic extract was loaded on a 2-mL affinity column packed with a complex Epoxi-activated Sepharose™ 6B (Amersham Pharmacia Biotech, Sweden) and acarbose (Sigma). The column was washed with buffer of 0.5 M NaCl/0.1 M NaOAc, pH 4.5, GA was eluted by 1.7 M Tris–HCl solution at pH 7.6, and protein containing fractions were dialyzed against distilled water.

Electrophoretic Methods

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli [19] using 10% gel in a Mini-Cell BioRad. Protein bands were revealed by silver staining [20].

Effect of Temperature and pH on Activity and Stability of the GA

Optimal activity pH and temperature and enzymatic stability have been described previously [14].

Irreversible Thermo-inactivation Kinetics

Purified mutant GA was incubated in 0.1 M NaOAc buffer, pH 4.5, at 65–80 °C with 2.5 °C intervals. Seven samples were withdrawn periodically at each temperature, cooled in a water bath, and enzyme activity was determined. Irreversible thermo-inactivation coefficients (k_d) for mutant and wild type were calculated by fitting linear regressions of ln (residual activity) versus inactivation time. The free energy of thermo-inactivation (ΔG) was calculated from ln (k_d/T) versus $1/T$ plots based on transition state theory [21]. The half-life ($t_{1/2}$) of enzymes was defined as the time at which after incubating at each temperature, enzyme activity was 50% of the original activity. The $t_{(1/2)}$ was also determined according to the equation: $t_{(1/2)} = 0.693/k_d$ [22].

Results and Discussion

Characterization of Thermostable Mutant GA from *A. awamori* Expressed in *S. cerevisiae*

The GA gene from the mutant was genetically characterized by DNA sequencing. From the mutant sequence, a total of four mutations were identified, all of which were identified in the catalytic domain. One mutation was silent (GGC→GGT), whereas three resulted in amino acid replacement: Ser54→Pro, Thr314→Ala, and His415→Tyr.

These changes, in the positions indicated, have not been described in the literature. However, Wang et al. [6] and McDaniel et al. [5] had residues where the Ser→Pro, Thr→Ala, and His→Tyr substitutions occurred in different positions in the gene. In all cases, there was increased thermostability.

The increased numbers of Pro residues often correlate with increased protein thermostability [5, 6, 23–26]. According to Gomes et al. [27], the increase in Pro concentrations helps thermostability due to the amino acid structure that has few possible configurations, reducing entropy of unfolding and thus stabilizing the protein.

Research papers have reported mutagenesis strategies to obtain a GA with higher thermostability, including by introducing extra disulfide bonds [5, 9, 28], reducing the backbone flexibility by introducing a Pro residue [5, 6, 25], reducing the number of possible conformations in the unfolded state of the protein by replacing Gly with others residues [9, 29], and through site-directed mutagenesis and random mutagenesis.

The effect of T314A and H415Yin stabilizing GA is hard to explain without digging deep into the current knowledge of GA structure and of protein folding theory, which is beyond the objective of this research. Further study correlating mutations of these residues in GA structure and protein folding are currently being conducted to help to explain the increased GA thermostability caused by these mutations.

The mutant GA exhibited optimum activity at pH 4.5 (Fig. 1a) and optimum temperature at 65 °C (Fig. 1b). It has been reported that fungal GAs act well in acid pH and generally at temperatures in the range of 50–60 °C [30]. The wild-type GA showed optimal activity at pH 3.5–4.0 and a temperature of 58 °C [14]. Anto et al. [31] described a GA from *Aspergillus* sp. HA-2 that exhibited an optimum temperature of 55 °C and an optimum of pH 5.0. GA from *Aspergillus oryzae* (OS1 1013) cultivated in submerged fermentation exhibited optimum activity at a temperature of 65 °C and an optimum of pH 4.5 [32]. Regarding stability, the enzyme kept 80% of its activity between pH 5.5 and 9.0 (Fig. 1a), and the enzyme was stable up to temperatures of 60 °C (Fig. 1b).

The irreversible thermo-inactivation of wild-type and mutated GA follows first-order kinetics. Figure 2 shows the effect of temperature on k_d for wild-type and mutated GAs. Other authors had also related first-order kinetics in their research [9, 24]. Kinetic parameters for irreversible thermo-inactivation of the mutant and wild-type GA are shown in Table 1. The half-life of the mutant GA was twice that of wild-type GA at all

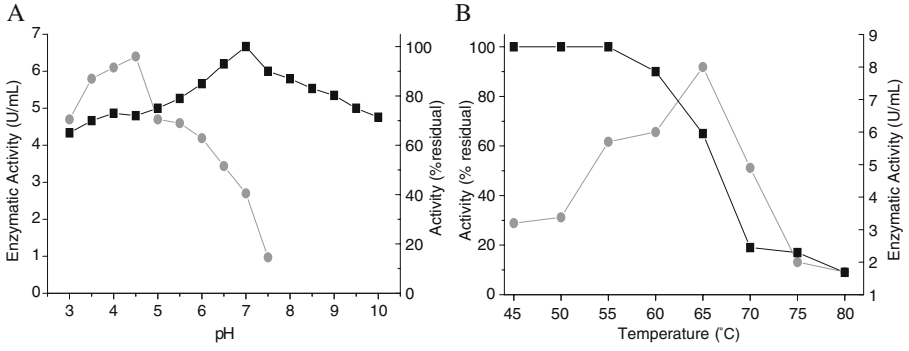

Fig. 1 Effect of pH (**a**) and temperature (**b**) on the pure mutant GA activity (symbols: *circle* optimum pH and temperature; *square* stability pH and stability temperature). Activity was measured with 0.5% soluble starch as substrate

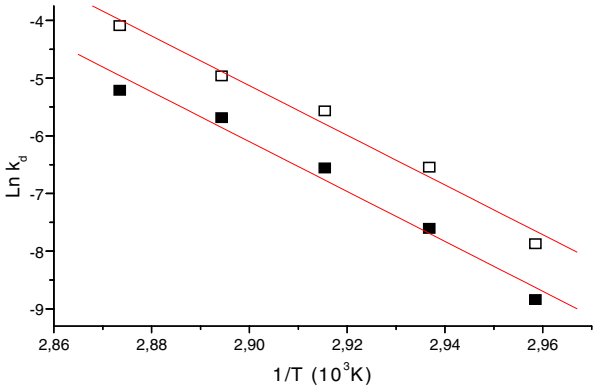

Fig. 2 Effect of temperature on thermostability of *A. awamori* mutated GA and wild-type GA. Symbols: *empty square* wild-type GA; *filed square* mutant GA

temperatures. At 65 °C, the half-life of the mutant was 76.1 and 30.2 min for wild-type GA. The free energy of thermo-inactivation (ΔG) for the mutant GA was higher than wild-type GA at all temperatures (Table 1).

High ΔG value indicates a thermostable enzyme, while a small value indicates a thermosensitive enzyme, as noted previously by other authors. Usually, proteins begin to unfold owing to the increased intramolecular motions caused by increasing temperature [9]. Thermal inactivation generally is caused by the rupture of non-covalent bonds followed by its unfolding. The thermo-inactivation process of GA is also caused by the incorrect conformation of the protein [33]. The stability of the enzyme's structure is the result of a delicate balance between several factors, such as the number of hydrogen disulphide bridges, hydrophobic interactions and the degree of molecular tangling, as well as the number and kinds of amino acids involved in this structure [34]. These closely related factors are what give the protein its stability. Any change that affects the integrity of the protein's form or fold can upset the balance. Moreover, proteins are fragile and easily disorganized by heat and other, even mild, treatments, and so small molecular alterations can generate significant changes to their stability.

Table 1 Kinetic and thermodynamic properties of mutant and wild-type GA of *A. awamori* expressed in *S. cerevisiae*.

Temperature (K)	$t_{(1/2)}$ (min^{-1})		ΔG (KJ mol^{-1})	
	Wild type	Mut	Wild type	Mut
338	30.2	76.1	106.1	102.5
340.5	8.0	23.2	104.6	101.6
343	3.0	8.1	103.5	100.7
345.5	1.6	3.6	102.3	99.7
348	0.6	2.1	101.0	98.8
350.5	0.6	1.5	100.0	97.8
353	–	0.8	98.6	96.8

– not detected

The combination of these three mutations increased the thermostability of mutant GA by 3.0 KJ mol^{-1} at 70 °C. Thus, the mutation of a given residue would change not only the conformational preference but also other interactions, possibly producing better thermostability.

Production and Purification of Mutant GA

Transformed yeast was grown in four different culture media. All substrates analyzed were good producers of the enzyme; however, the production of GA was stimulated differently, according to the source of starch used. The best substrates for mutant GA production were potato starch and soluble starch, yielding enzyme productions of 6.6 U/mL (20.9 U/mg) and 6.3 U/mL (22.5 U/mg), respectively. The cassava starch was also a good substrate for GA mutant production (5.9 U/mL, 12.3 U/mg), followed by corn starch (3.0 U/mL, 16.6 U/mg). For all the substrates evaluated, the highest enzyme production was observed after 96 h of fermentation (Fig. 3). In previous research, the wild-type GA of *A. awamori* expressed by *S. cerevisiae* was also produced in different substrates. The best substrate for this enzyme production was also the potato starch, followed by soluble starch, cassava starch, and corn starch [14]. The mutation did not affect the protein secretion nor the production of GA studied in this research, although some researchers reported that mutations could affect the enzyme conformation secreted and thus influence its activity [12, 35–38]. The enzyme activity and secretion might also be affected by a high quantity of glycosylation [39–40].

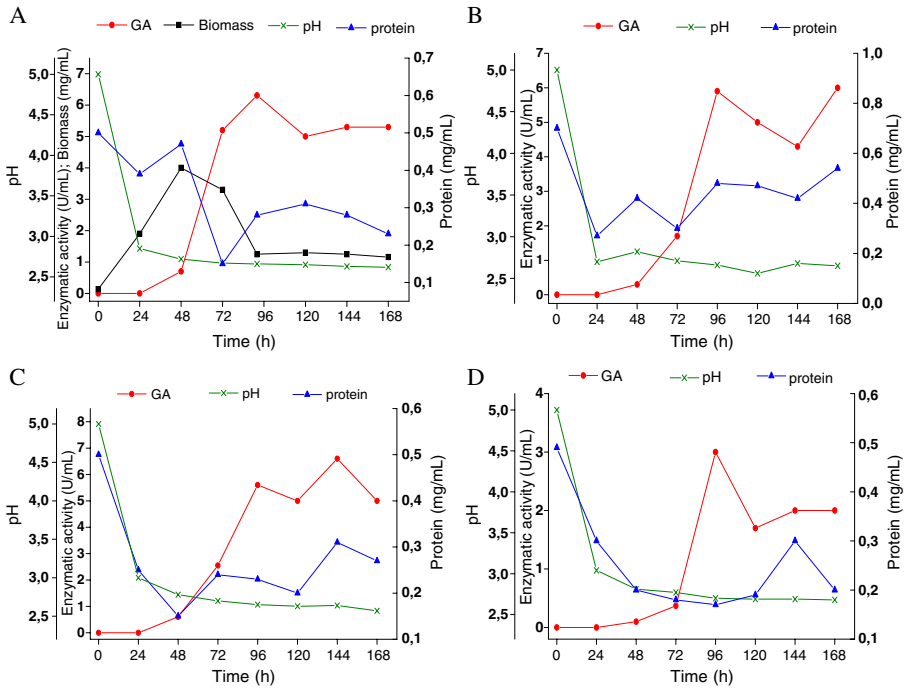

Fig. 3 Mutant GA production by yeast *S. cerevisiae* in medium with starches from different sources. **a** Soluble starch; **b** Cassava starch; **c** Potato starch; and **d** Corn starch. To determine the cellular mass, it was dried at 60 °C until it reached a constant weight

Our results are significant when compared to others in the literature. For example, Chen et al. [21] reported a wild-type GA of *A. awamori* production by *S. cerevisiae* of 0.31 U/mL after 60 h of fermentation and a mutant Asn 182→Ala GA production of 0.50 U/mL. Both enzyme productions were lower than those observed in the present work.

Other authors also reported wild and recombinant GA productions lower than those detailed in this work. A hybrid strain CL-9 of *Saccharomyces diastaticus* produced GA with 1.06 U/mL in a medium containing glucose and starch [13]. The GA activity of *S. cerevisiae* ATCC 9763 expressing the *GAM1* gene from *Debaryomyces occidentalis* was 0.92 U/mL in a medium with 3% starch and 2% glucose [41]. The GA activity from *Saccharomycopsis fibuligera* DSM-70554 was 0.65 U/mL after 48 h of fermentation in a medium containing starch as substrate [42]. The GA activity expressed by immobilized recombinant *S. cerevisiae* strain C468 containing the plasmid pGAC9 was 0.16 U/mL [43]. From these data, we can infer that *S. cerevisiae* is an efficient GA producer.

Regarding biomass production in a medium with soluble starch, the highest value (4.0 mg/mL) was observed after 48 h (Fig. 3a). Biomass decrease was followed by an increase in GA secretion, and this feature has also been observed in other research [13]. The highest biomass production by *S. diastaticus* (CL-9) was 9.3 mg/mL when cultivated in a medium containing glucose as the carbon source and 2.0 mg/mL in a medium containing starch [13]. The strain *S. fibuligera* DSM-70554 was grown on cassava starch for 48 h and had a biomass estimated at 2.3 mg/mL [42]. Although some studies showed a higher biomass yield, this is not reflected in a greater GA activity of these strains.

Regarding protein concentration, it is higher at the beginning of cultivation probably due to the presence of amino acid histidine in the culture medium. The culture medium pH was highly reduced, decreasing to a limit of 2.6 at the end of cultivation. This intense

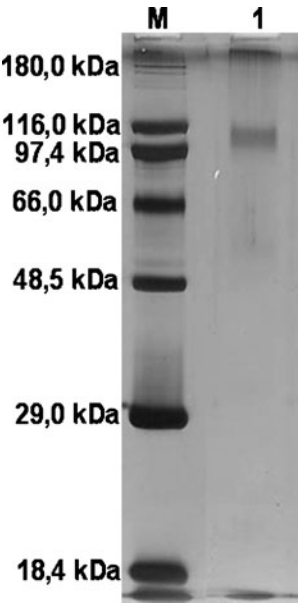

Fig. 4 SDS-PAGE of thermostable GA of *A. awamori* expressed in *S. cerevisiae*. *M* standard molecular weight markers; *1* mutant thermostable GA

acidification is the result of nutrient consumption and the release of metabolites during fermentation. This characteristic was also observed for the wild-type enzyme [14]. Yeasts, especially *S. cerevisiae*, tolerate a wide pH variation (2.5–8.5), but its optimum growth pH is 4.5. However, the intracellular pH seems to be independent from the extracellular one, which is maintained between 6 and 7 [44], since the internal acidification in yeast is controlled by H+ATPase of the plasma membrane, allowing the alkalinization of the intracellular medium [45]. Besides, to avoid stress effects that could influence the metabolism, yeast has a response mechanism that acts to reduce the intracellular accumulation of weak acids at potentially toxic levels [46–48]. However, extreme pH can cause cellular stress and put pressure on the ATPase system, decreasing the energy available for growth and other essential metabolic functions [49, 50].

The molecular mass of the pure enzyme was determined by SDS-PAGE and estimated as 100 kDa (Fig. 4). This molecular mass is in the range found for the majority of the fungal GAs that varies between 26 and 112 kDa [4, 51]. Flory et al. [35] reported mutant GA with a similar molecular mass. The purified GA from *Aspergillus niger* (CCUG 33991) was reported to have a molecular mass of 90 kDa [52]. GA from *A. niger* AB4.1 (pgpdAGLAGFP) purified for affinity chromatography had a molecular mass of 100 kDa [53].

A summary of the purification procedure is shown in Table 2. The high purification factors observed in the present work can be explained by the low yield values. The high specific activity is probably due to the extreme purity of the enzyme (Table 2).

Conclusions

The GA mutation using error-prone PCR produced three amino acid alterations in the protein structure (Ser54→Pro, Thr314→Ala, and His415→Tyr), and these alterations contributed to a significant increase in thermostability when compared to the wild-type enzyme. The mutant GA showed an increase of 7 °C in the optimum temperature and an increase of 3.6 KJ mol^{-1} in the free energy of thermo-inactivation (ΔG) at 65 °C and 1.8 KJ mol^{-1} at 80 °C in relation to the wild type. The mutant GA has a half-life of 76.1 min at 65 °C, a value twice that of the half-life of the wild-type enzyme. Mutant GA expressed by *S. cerevisiae* produces better enzymatic activity than when potato starch was used as the substrate, and the mutation in this GA did not reduce the capacity of the yeast to produce high levels of GA.

Table 2 Purification of mutant GA from *A. awamori* expressed by *S. cerevisiae*.

Purification step	Volume (mL)	GA activity		Protein		Specific activity (U/mg)	Yield (%)	Purification factor
		(U/mL)	(U total)	(mg/mL)	(mg/total)			
Crude extract	400	2.5	1,000	0.30	120	8.4	100	1
Concentraded extract	16	52.9	846.4	0.49	7.8	107.9	84.6	12.9
Affinity chromatography	5.0	5.0	25.0	0.004	0.02	1250	2.5	150.6

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